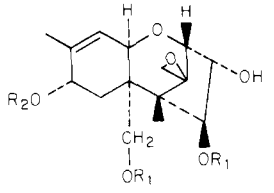
Table IV

Compound	Isolated, %	Analyses,[a] %
1	95	100
2	98	100
3	96	100
T-2 toxin[b]	96	100
T-2 tetraol[b]	96	100

[a] Determined by gas chromatographic analysis. [b] See structures in text.



T-2 toxin, $R_1 = CH_3CO-$; $R_2 = (CH_3)_2CHCH_2C(=O)$
T-2 tetraol, $R_1 = H$; $R_2 = H$

liliters of an aqueous methanolic solution of sodium acetate (10% w/v) was added to a solution of 5 mg of **2** in 2 ml of methanol. The pH of this mixture was brought to 9.5 by the addition of a drop of 0.01 *N* NaOH and the progress of solvolysis followed by TLC at 30-min intervals. At the end of 4 hr, the mixture was resolved on TLC ($CHCl_3$–MeOH, 90:10) and consisted of 75% of **1**, 20% of **2**, 3% of **3**, and 2% of an unidentified component (probably an epimer or an isomer of **2**).

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Characterization of Proteins and Allergens in Germinating Castor Seeds by Immunochemical Techniques

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Phosphate buffer extracts (pH 7.0–7.5) of mature ungerminated castor seeds, *Ricinus communis* L., contained at least seven major antigens detectable by immunoelectrophoretic analysis (IEA) against immune serum for the total castor seed proteins. The classical CB-1A allergen was identified in the total protein extract by using immune serum containing CB-1A antibodies. By IEA, specific proteins in certain allergen fractions were identified, and changes in the major proteins of dormant seed occurring after germination were easily detected. The CB-1A allergen contained a major antigen and at least two minor antigens. Some of the major proteins of dormant seeds changed significantly after 6 days' germination, but the major CB-1A antigen was still present.

Castor seed, *Ricinus communis* L., has long been an important industrial oilseed because of its unique and high oil content. It contains 50–60% oil and 18–20% protein. World production of the oil in 1971 was 325000 metric tons (Agricultural Statistics, 1972). The residue (over 100000 metric tons of defatted meal, or pomace) was generally used as fertilizer rather than as animal feed because it contained ricin (a toxalbumin), several potent allergens, and the alkaloid ricinine (Coulson et al., 1960; Waller and Negi, 1958). Attempts to detoxify castor seed meal have been reported (Mottola et al., 1972) but the products are still not considered major sources of protein for feed uses.

The allergens from defatted meals of mature castor seeds have been studied extensively. Berrens (1971) compared the electrophoretic migrations of 26 different allergens and concluded that atopic allergens were fast-moving anodic glycoproteins, but the basic allergenic proteins of castor seed were an exception to this general pattern. Layton et al. (1961) had examined the classical CB-1A allergen by paper electrophoresis and resolved it into six or more components at pH 8.0. Spies (1974) recently reviewed published research on allergens of dormant castor seeds, but few of the reports included the total proteins extracted from castor seeds.

Mourgue et al. (1958) separated the total proteins of

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dormant castor seeds, extracted at pH 6.4–7.5, by paper electrophoresis into seven fractions, four that migrated to the cathode and three to the anode. More recently, Sobolev et al. (1972) used gel electrophoresis and immunodiffusion to study the synthesis of storage proteins in developing (ripening) castor seeds. They showed that the storage proteins were absent at early stages of seed formation and appeared only after the seeds had reached a certain degree of maturity.

The lack of available information on total proteins of castor seeds and on changes in the proteins of germinating seeds prompted this investigation. The purpose of this report is to enumerate the antigenic constituents of dormant castor seeds, to identify certain allergens in extracts of the seeds, and to determine, for germinating seeds, the changes occurring in proteins, particularly the CB-1A allergen and the major storage globulin.

MATERIALS AND METHODS

Germination of Seeds. Castor seeds, Baker 296 variety, were a gift from D. S. Bolley and W. E. Domingo, Baker Castor Oil Co. Each 20-g lot of seeds was placed between two layers of absorbent paper which were then rolled up and placed in a tall beaker with a small amount of water. A second beaker was placed on top to maintain a moist atmosphere for 3, 6, and 10 days in a 30°C cabinet in the dark.

Extraction of Proteins from Seeds. For ungerminated seeds, 50 g of dry seeds (minus seedcoats removed manually with a scalpel) and 100 ml of the 0.05 *M* phosphate buffer, pH 7.0–7.5, containing cysteine and ethylenediaminetetraacetic acid (EDTA) used for preparation of the castor lipase (Altschul et al., 1963) were homogenized in a food blender for 1 min and then centrifuged 40 min at 3000 rpm. The fatty layer was removed by a spatula. The aqueous phase was transferred to clean centrifuge tubes and recentrifuged to remove residual fat. The aqueous supernatant was decanted carefully through filter paper (general yields were 65–70 ml) and used directly for immunoelectrophoretic analysis (IEA), or portions of the extract were dialyzed against water and freeze-dried. After determining net yield of protein per milliliter of filtrate, the residue was reconstituted in buffer for IEA.

For germinated seeds, the entire seedlings (root, endosperm, and shoot) of the 20-g lots were homogenized in 60 ml of buffer in a blender, and total protein extracts were prepared as described for dry dormant seeds.

Preparation of Proteins and Allergens. Samples of CB-1A and CB-1C used for the preparation of antisera were generously provided by Dr. J. R. Spies. CB-1A for IEA was prepared in our laboratory from mature castor seeds by the method of Spies and Coulson (1943). (CB-1C is chemically the same as CB-1A, but carbonate is used to remove lead ions in preparing CB-1C and H_2S is used for CB-1A.) CB flour, a white powder, was prepared as follows. Hand-hulled castor bean meats were extracted with hot hexane, then with boiling ethanol. The fat-free meal was boiled for 1 hr in water to inactivate ricin, and the aqueous solution freed of all solids was lyophilized to yield the CB flour. CB-WU was prepared by the method of Layton et al. (1961). This is obtained by water extraction of CB flour, boiling to inactivate ricin, and then separating the protein fraction by several precipitations from aqueous ethanol at 0°C.

Immunochemical Procedures. The potent toxins and allergens in castor seeds presented an additional problem. Although Coulson et al. (1950) showed that antiserum could be prepared with extracts containing ricin by first building up the animal's tolerance to ricin and then administering larger doses to induce antibody formation, we wished to avoid the lethal effects of ricin on the animals and a potential hazard to sensitive laboratory personnel during the experiments. Therefore, ricin (and other heat-sensitive antigens) in the defatted flours or extracts were first destroyed by heat before injecting into rabbits and for subsequent experiments. Flours or extracts were mixed with Freund's complete adjuvant and injected into rabbits for a time sufficient to yield high antibody titers, over long periods and/or in small doses. The rabbits were then bled to obtain immune sera for CB flour, CB WU, CB-1A, and CB-1C allergens.

IEA was conducted according to Grabar and Williams (1953) with an LKB Immunophor (3276BN) at 200 V, at room temperature (22°C) for 2 hr, and with 1.5% Ionagar gel in 0.25 *M* Veronal buffer (pH 8.6). The slides were stained with a solution of Amido Schwarz in methanol containing 5% acetic acid in water.

Table I. Wet Weight of Seedlings and Yields of Protein Fractions Extracted from Castor Seeds (Values Are Those from 20-g Lots of Dry Seeds)

Sample description	Wet wt germinated seeds, g	Total protein[a] yield/20-g lot of seeds, g	Yield total proteins[a] per single seed, mg
(CB_0-1) dry dormant seeds		1.208	17.7
(CB_0-2) dry dormant seeds		0.959	14.9
(CB_3) 3-days germinated seeds	24.4	0.927	21.5
(CB_6-1) 6-days germinated seeds	66.2	1.574	23.3
(CB_6-2) 6-days germinated seeds		1.568	23.0
(CB_{10}) 10-days germinated seeds	108.3	0.580	9.7

[a] Soluble proteins (including ricin) extracted with buffer, dialyzed vs. water, freeze-dried.

RESULTS AND DISCUSSION

Total Proteins in Castor Seeds. Table I shows that the wet weights of 20-g lots of seeds after germination increased slowly up to 3 days, then rose rapidly and linearly up to 10 days. The weights of total proteins (including ricin), after dialysis and freeze-drying, did not seem to follow as consistent a pattern. The increased wet weight of 10-day seedlings may have been due to water and/or carbohydrate but no analyses were made to verify this.

Figure 1 shows the IEA of the protein extracts against different antisera. Slide 1 shows four major (C, D, E, and F) and at least two minor (X and a faint arc at lower right) cathodic antigens, plus two major anodic antigens (A, B) in a total protein extract of castor seeds. Mourgue et al. (1958) separated their total castor proteins into four cathodic and three anodic bands by paper electrophoresis. Combining polyacrylamide gel electrophoresis and immunodiffusion, Sobolev et al. (1972) resolved mature castor seed extracts into about nine electrophoretic bands in gels, four or five which were major-staining ones. By immunodiffusion, the slowest moving major globulin of their partially ripe and ripe (mature) castor seeds was found to be identical with the crystalloid protein of the protein bodies.

Identification of Specific Proteins. Some proteins can be further characterized by using antibodies to the

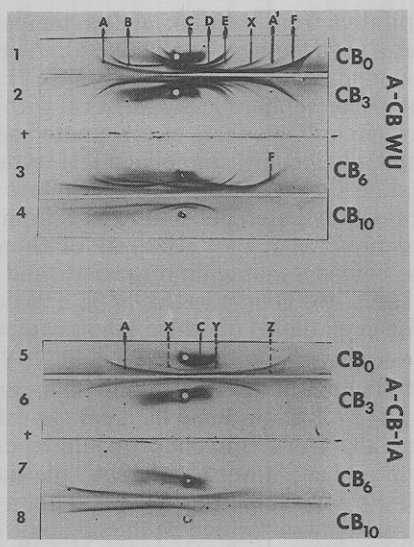

Figure 1. Immunoelectrophoretic analysis of total protein extracts of dormant and germinated castor seeds. Extracts tested: CB_0, dormant (ungerminated) seeds; CB_3, CB_6, CB_{10}, seeds germinated for 3, 6, and 10 days, respectively. Antisera: A-CB WU (total proteins minus ricin from dormant seeds), A-CB flour (total proteins minus ricin from defatted dormant castor seed meal), A-CB-1A (antibodies to CB-1A allergen preparation), and A-CB-1C (similar to CB-1A, minus part of the carbohydrate moiety) are described under Materials and Methods. Conditions for IEA: extracts made up to 10 mg/ml for testing; 1.5% Ionagar in 0.037 ionic strength, pH 8.6 Veronal buffer, used for coating slides; IEA for 2 hr at 200 V.

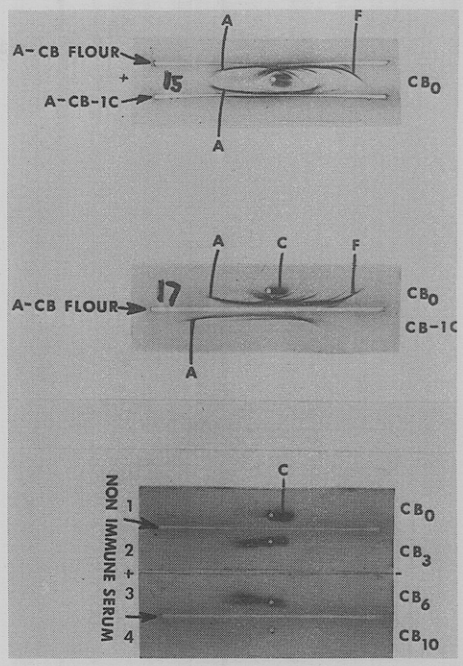

Figure 2. Comparison of ungerminated castor seed proteins and the CB-1C allergen by immunoelectrophoretic analysis. Antisera: A-CB flour and A-CB-1C, same as in Figure 1; nonimmune serum, normal serum containing no antibodies to castor proteins. All other designations and conditions same as in Figure 1.

total proteins (minus ricin) of dry mature seeds and to the CB-1A allergen. Antigen A, appearing as a rather long arc in slide 1 (vs. antitotal protein), also appears to be the major arc in slide 5 (vs. anti-CB-1A immune serum), Figure 1. The CB-1A preparation was resolved into six major and two minor bands by paper chromatography by Layton et al. (1961) and into six bands by electrophoresis on cellulose acetate by Spies and Barron (1966). The latter authors found that four of these bands, representing 85% of the recovered materials, had the same antigenic specificity. Common antigenic specificity in chemically distinct fractions was also demonstrated after ion exchange fractionation of CB-1A (Spies and Coulson, 1964) and after examination of the number and homogeneity of the isolated antigens of fraction CB-1A (Spies, 1967). Because of its very elongated shape, precipitin band A might correspond to several of the electrophoretically distinct constituents found in CB-1A by Layton et al. (1961) and by Spies and coworkers (Spies and Coulson, 1964; Spies, 1967).

In addition to this electrophoretic heterogeneity of the major antigen, A, there are several other antigens in the allergen fraction, shown by IEA of the CB_0 extract using the anti-CB-1A immune serum (Figure 1, slide 5) and the anti-CB-1C immune serum (Figure 2, slide 15), and by IEA of the CB-1C fraction with the anti-CB flour immune serum (Figure 2, slide 17).

In the crude extract, antigen B is readily detected using the anti-CB WU immune serum but not with anti-CB-1A (Figure 1), suggesting that B is not a constituent of the allergen fraction. This is confirmed by IEA of the crude extract, CB_0, vs. anti-CB-1C and of the allergenic fraction, CB-1C, using anti-CB flour immune serum (Figure 2).

The diffuse arc, C, easily identified in extracts of ungerminated and 3-days germinated seeds, and to a lesser extent in 6-days germinated seeds, vs. anti-CB WU (Figure 1), is also detected when a nonimmune serum (taken before immunization) is used (Figure 2). C is, therefore, not an immunoprecipitin band. The nature of this material that is not present in the Spies CB-1C allergen preparation (Figure 2, slide 17), and which progressively disappears upon germination, is still unknown.

In Figure 1, slides 1 and 5 show that antigen D was detected in the total castor proteins using the anti-CB WU immune serum but not with the anti-CB-1A. Nevertheless, the similarity of the electrophoretic mobilities between arc D, detected with anti-CB WU serum (slide 1), and the faint arc Y, detected with anti-CB-1A serum (slide 5), suggests that D may possibly be a minor constituent of the allergen fraction. However, this can only be confirmed by additional cross-reaction tests with the CB-1A fraction and anti-CB WU immune serum.

Antigen E (Figure 1) seems to be a major constituent, similar to B and D, in the ungerminated seed extract. But unlike B and D, this protein was still present in extracts of 10-day seedlings (slide 4), and did not seem to have antigenic determinants common to the CB-1A allergen. E was not detected in the CB_0 extract with anti-CB-1A immune serum (slide 5) and did not occur in the CB-1C fraction vs. anti-CB flour immune serum (Figure 2). The lack of complete catabolism within 10 days' germination suggests that E is not a storage protein but may be a major functional protein found in both the seed and the growing seedling.

F was the most cathodic antigen and formed the largest arc in total protein extracts of the castor seed (slide 1); it was not detected with the anti-CB-1A immune serum (slide 5). Based upon the following evidence, F is identified as the major storage globulin in castor seed protein bodies photographed by electron microscopy in our laboratory (Ory et al., 1968) and appears to be identical with the crystalloid protein found by Sobolev et al. (1972) using gel electrophoresis and immunodiffusion. Protein F forms a continuous precipitin band and seems to be composed of at least three types of molecules (possibly subunits) having common antigenic determinants, but differing in their

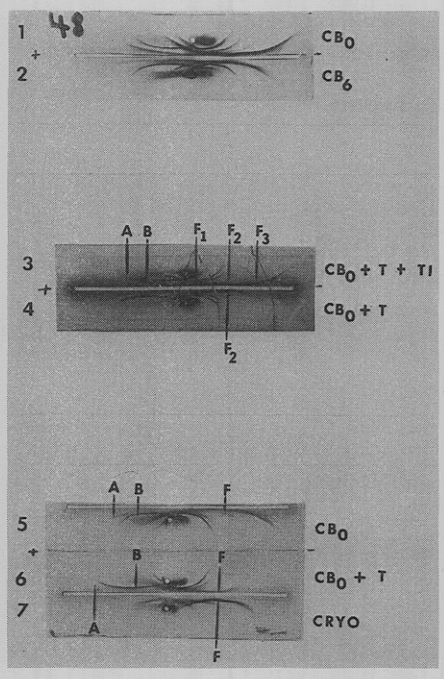

Figure 3. Immunoelectrophoretic analysis of total castor seed proteins before and after germination, after trypsin treatment, and after cryoprecipitation. Trypsin treatment (CB_0 + T), 0.1% for 1 hr; plus trypsin inhibitor (CB_0 + T + TI), and cryoprecipitate (CRYO). All other designations and conditions are the same as in Figure 1.

electrophoretic mobilities. In a similar study of peanut proteins by IEA, Daussant et al. (1969a,b) identified α-arachin as the major reserve protein in the protein bodies and detected changes in mobility of α-arachin after only 1 day of germination. Like protein F of castor seed, α-arachin retained its antigenicity during germination. Its precipitin band, however, became longer and more extended than did arc F (Figure 1, slide 3).

By IEA, Daussant et al. (1969a) also showed that treatment with 0.1% trypsin increased the electrophoretic mobility of α-arachin. Treatment of castor proteins with 0.1% trypsin indicated that protein F was also sensitive to attack (Figure 3). Slides 1 and 2 show the changes in F during normal germination for 6 days. The total protein extract treated with trypsin that had been inactivated with (soybean) trypsin inhibitor (slide 3) appeared to be the same as that which was not treated (slide 1). However, treatment with "active" trypsin distinctly affected protein F. Unlike the electrophoretic band of peanut protein, arachin, which elongates upon treatment with trypsin, that of castor protein F seems to become shorter and diminishes in concentration. Comparisons of the effects of germination (slide 2) and trypsin (slide 4) on the three molecular constituents (F_1, F_2, F_3, in slide 3) of protein F show that the most anodic (F_1) and most cathodic (F_3) constituents were metabolized (proteolytic degradation) more rapidly than the constituent of intermediate mobility (F_2). (These experiments also show that antigen, A, of the CB-1A allergen and protein B of the total proteins underwent anodic shifts in mobility after trypsin treatment.)

Using gel electrophoresis, Sobolev et al. (1972) identified the crystalloid protein of developing castor seed protein bodies as a slow-moving globulin of large molecular weight, forming two–four overlapping bands (different molecular constituents) near the origin. Gel electrophoretic analyses of peanut proteins by Cherry et al. (1973) showed that α-arachin is also a slow-moving globulin of large molecular weight, forming two–three overlapping bands, with about the same R_f as Sobolev's crystalloid protein. α-Arachin was precipitated from solution at low temperatures, a "cryoprotein", by Daussant et al. (1969b). A cryoprotein was also found in castor seeds (Figure 3, slide 7). Its major component is electrophoretically identical with antigen F of total protein extracts from ungerminated seeds (slide 5) and to partially metabolized antigen F of trypsin-treated proteins (slide 6). Also, the cryoprecipitate is not as electrophoretically heterogeneous as is F from ungerminated seed proteins (CB_0). Because of these striking similarities between α-arachin of peanuts and protein F of castor seeds, we conclude that F is a major reserve protein (the crystalloid) of castor seed protein bodies.

Unknowns, X of the total proteins and X, Y, and Z of the CB-1A allergen in ungerminated seeds, could not be identified (Figure 1, slides 1 and 5). Only the cathodic end of unknown arc X of CB in slide 1 could be detected in the total proteins and it was not the cathodic end of either arc A or B. By superimposing slides 5–6 upon slides 1–2 (Figure 1), arc A' was verified as the cathodic end of arc A, based upon the shape and positions of the arcs. The presence of A was confirmed by the use of anti-CB-1A immune serum (slides 5–6), which produced fewer precipitin arcs than anti-CB WU.

Unknowns X, Y, and Z of the allergen (slide 5) were minor antigenic components of the heterogeneous CB-1A preparation resolved into six or more bands by Layton et al. (1961). Unknown X in CB-1A differed from X in the total protein extract and was not detected with anti-CB WU. Although we have labeled arc Z as a separate unknown in an effort to correlate it with one of the bands resolved by Layton et al., it might be the cathodic end of arc X in slide 5.

Changes in Proteins during Germination. Variations in electrophoretic mobilities and losses of antigenicity were observed for several constituents of castor seed extracts after 6–10 days' germination. Antigen A of the CB-1A allergen preparation (Figure 1, slides 5–8) shifted anodically during germination but maintained a significant proportion of its antigenic determinants (slide 8). This suggests that a change has occurred in its conformation or spatial configuration and that the carbohydrate-rich components (glycoproteins) of CB-1A are not hydrolyzed as readily as the low carbohydrate fractions. Electrophoretic anodic shifts occurring in storage proteins upon germination without apparent modifications in their antigenicity have been reported for phaseolin in *Phaseolus* (Kloz et al., 1966), for the 11S and 7S proteins in soybean (Catsimpoolas et al., 1968), and for α-arachin in peanut (Daussant et al., 1969a). These shifts might occur by progressive deamidation of glutamine and asparagine in the proteins. The intensities of precipitin arcs B, D, and E of the total extract (Figure 1, slide 1; Figure 3, slides 1 and 2) also decreased up to 6 days germination. After 10 days, from these three arcs, only E was still present and, like A, had become more anodic, giving rise to a modified but still antigenic form (Figure 1, slides 3 and 4). Although changed after germination, E was not altered by trypsin (Figure 3, slides 3–6). The unidentified antigen, Y, of the CB-1A allergen (Figure 1, slide 5) disappeared after 3 days (slide 6). If proteins D (slide 1) and Y (slide 5) are related, the more rapid disappearance of Y (slide 6) compared to D (slide 2) suggests that the antigenic site common to both proteins was completely degraded within 3 days.

The effects of rapid catabolism of the major protein, F, during 6 days' germination (Figure 1, slides 1–3) were similar to those induced by trypsin hydrolysis (Figure 3, slides 4 and 6). By 10 days, F was completely absent. Between 0 and 6 days, the large arc formed by the three

constituents in ungerminated seeds was transformed into one smaller arc with about the same electrophoretic mobility as that of the middle constituent (F_2) of the original protein. The most anodic and most cathodic constituents (F_1 and F_3) were catabolized enough to lose their antigenicities. The two components, F_1 and F_3 (represented as two or more bands by Sobolev et al. (1972) for the castor crystalloid protein), were more labile than the protein of intermediate mobility, F_2, which seemed to predominate. This was noted after 6 days' germination or after treatment of the proteins with trypsin. The reasons for this phenomenon are unknown and higher purification of protein F and examination of the structures of its constituents will be required.

In summary, a total protein extract of ungerminated castor seeds contained at least seven antigens of which the CB-1A allergen, the major storage protein, and four cytoplasmic (or subcellular) proteins were identified. Germination of the seeds caused an anodic shift in the CB-1A major antigen and a decrease in the most anodic and cathodic portions of the major storage protein, F. These changes could have been due to a configurational change caused by increased exposure of surface-charged groups on the proteins, but without loss of antigenicity or complete loss of antigenic structure. After germination some proteins were no longer detectable by IEA; but even after 10 days, proteins extracted from the seedlings still contained a significant amount of the major CB-1A antigen.

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Isolation of *Gonyaulax tamarensis* Toxins from Soft Shell Clams (*Mya arenaria*) and a Thin-Layer Chromatographic–Fluorometric Method for Their Detection

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A thin-layer chromatographic–fluorometric method for the detection and determination of *Gonyaulax tamarensis* toxins in column chromatography fractions is described. Fractions are chromatographed on silica gel plates and the plates sprayed with 1% hydrogen peroxide, heated, and scanned in a fluorometer. By this means 40–400 ng of the various poisons could be determined. Monitoring with this procedure, *G. tamarensis* major toxin was separated into major toxins H and L, with approximate potencies of 1800 and 4200 mouse units per mg, respectively. At pH 4.7 major toxin H was unaffected, even after heating, while L appeared partially converted to H. At pH 8.2 at 16°C major toxin H was unchanged, while L gave H and a small amount of material which corresponded in R_f to saxitoxin. At pH 8.2 heating destroyed both toxins. The fluorescence properties of major toxins H and L and saxitoxin after hydrogen peroxide treatment also suggest similarity in their structures.

Paralytic shellfish poisoning (PSP) is a severe form of food intoxication which occurs in widely scattered areas of the globe (Halstead, 1965). The poison is produced by certain species of marine dinoflagellates which normally make up only a very small portion of the plankton upon which the shellfish feed. Occasionally, however, these toxic dinoflagellates bloom, reaching concentrations as high as 40000 cells/ml. This condition is often called a "red tide". Shellfish, themselves unaffected, filter the dinoflagellates from the water, effectively concentrate the poison, and become toxic to humans and other animals that feed on them.

Shellfish from areas of the United States and Canada affected by outbreaks of PSP are continually monitored for the presence of poison using a modification of the bioassay (mouse test) first employed by Sommer and Meyer in 1937 (see Halstead, 1965). Although chemical (McFarren et al., 1958, 1959) and serological tests (Johnson and Mulberry, 1966) for the estimation of paralytic

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